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Title: Deoxynivalenol degradation in wheat kernels by exposition to ammonia
vapours: A tentative strategy for detoxification.

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Abstract

Deoxynivalenol (DON) is a mycotoxin produced mainly by *Fusarium* species and occurs predominantly in cereal grains such as wheat. Due to its toxic effects, in the European Union DON content in unprocessed cereals and processed cereal-based products for human consumption has been regulated, and recommended maximum limits have been established for animal feed. In this study, a method for degrading DON on wheat kernels, by exposition to ammonia (NH₃) vapours, was optimized. Results have shown that with a simple treatment with ammonia vapours at 90°C (for two hours), degradations higher than 75% were achieved in kernels affected by a moderated contamination up to 2000 µg/kg DON. The study of the reaction between DON and NH₃ allowed us to tentatively establish the structure of possible degradation products. In addition, *in silico* evaluation indicated, in general, lower toxicity and biological effects for the degradation products than for DON.

Keywords: DON; *Fusarium* toxin; Mycotoxin; chemical treatment; Detoxification; Degradation products

1 Introduction

Nowadays, the need to control toxins in food (and feed) products is generalized over the world. In the case of mycotoxins, due to their high toxicity, food producers establish strict controls for the raw materials used for food production. Deoxynivalenol (DON) is a trichothecene produced mainly by *Fusarium* species and occurs predominantly in cereal grains such as wheat, barley, oats, rye and maize. DON is the most commonly detected trichothecene in cereal grains and is also frequently found in higher concentrations than other mycotoxins (Shi, Schwab, & Yu, 2019; Stanciu et al., 2019). The consumption of contaminated wheat and wheat-based products appears to be a significant source of human exposure to DON (Khaneghah, Martins, von Hertwig, Bertoldo, & Sant'Ana, 2018). The main concern regarding DON is its chronic toxicity, which can lead to weight loss, anorexia and loss of nutritional efficiency (Payros et al., 2016; Pestka, 2007). DON is also considered a major cause of economic losses in animal husbandry (Morgavi & Riley, 2007). The European Union (EU) regulation for contaminants in food products limits the DON content in unprocessed cereals and processed cereal-based products between 500 and 1750 µg/kg, lowering the limit to 200 µg/kg in case of processed cereal-based foods and baby foods for infants and young children (European Community, 2006b). Recommendations on DON limits for products intended for animal feeding are also established in the EU, ranging from 900 to 12000 µg/kg (European Community, 2006a).

Although in the food industry DON contaminated batches of raw materials are usually discarded, there is an ongoing research focused on the effects of common and novel food processing techniques on the DON content of food

products, especially in cereals and derived products (Karlovsy et al., 2016). In relation to the usual food processing treatments, much attention has been paid to thermal processing, particularly to baking (Wu, Kuča, Humpf, Klímová, & Cramer, 2017). DON degradation has also been studied by other thermal treatments like frying (Moazami Farahany & Jinap, 2011), boiling (Vidal, Bendicho, Sanchis, Ramos, & Marín, 2016) or steaming (Cenkowski, Pronyk, Zmidzinska, & Muir, 2007).

Although for the most common use of cereals and flours a thermal treatment could be acceptable, sometimes the properties of the raw product are desired to remain unchanged. In this way, studies using approaches like UV or pulsed light for degrading DON in cereal grains have been carried out (Chen et al., 2018; Murata, Yamaguchi, Nagai, & Shimada, 2011; Popović et al., 2018). However, taking into account the contamination heterogeneity of cereal grain batches and the limited effect of light treatments in solids (zone of light incidence and shadow zones), these approaches seem more suitable for the treatment of liquid and more transparent food products. Similar limitations can be attributed to other modern techniques such as the cold plasma (Hojnik et al., 2019; Ten Bosch et al., 2017).

As an alternative to these “non-thermal” techniques, the use of chemical compounds in gas state would allow a more intense contact with the product, increasing in this way the effectiveness of the treatments. Wang et al. (2016) applied ozone on 11.8% moisture content wheat kernels, achieving a 42.0% DON degradation in 1 h. Although these results are interesting, the use of ozone presents some important limitations related with the “in situ” generation requirements and that it is not possible to store it. In this sense, a more simple

78 approach to treat wheat grains could be the use of ammonia (NH_3), that can be
79 easily stored as gas (in pressurized bottles) or in water solution as ammonium
80 hydroxide. In addition, regarding the safety of the use of ammonia treatments in
81 food processing, it is important to say that ammonium ion is present in the
82 common rising agent ammonium carbonate (E-503), an additive allowed by the
83 EU in processed cereal-based foods (European Community, 2008).

84 The use of ammonia has been evaluated for degrading mycotoxins, aflatoxins
85 being the most studied ones. Ammonia aflatoxin degradation has been studied
86 in matrices such as corn, wheat, cotton and peanut (Brekke, Peplinski, &
87 Lancaster, 1977; Chelkowski et al., 1981; Gardner Jr., Koltun, Dollear, &
88 Rayner, 1971; Mann, Codifer Jr., Gardner Jr., Koltun, & Dollear, 1970; Weng,
89 Park, & Martinez, 1994). However, studies with other mycotoxins are really
90 scarce. In the case of DON, only very preliminary studies have been published.
91 Thus, Young, Subryan, Potts, McLaren, & Gobran (1986) treated wheat with 5%
92 ammonium hydroxide solution (600 ml/kg wheat), obtaining a 35% DON
93 reduction. The effectiveness of the ammoniation process depends on the
94 treatment conditions (temperature, pressure, time) and also on the
95 contaminated product (Samarajeewa, Sen, Cohen, & Wei, 1990). Among those
96 factors, temperature has considerable importance. Young (1986) observed that
97 treating DON-contaminated corn with ammonium carbonate was more efficient
98 at 132°C (92% degradation) than at 100°C or 70°C (86% and 12% degradation,
99 respectively). Other authors have also previously observed that detoxification of
100 other mycotoxins by ammoniation is more effective when temperatures are
101 increased (Brekke et al., 1977; Chelkowski et al., 1981; Weng et al., 1994).

Therefore, the aim of this work was to study the DON degradation on wheat kernels by ammoniation, optimizing the processing conditions of temperature and ammonia concentration. In addition, studies on the impact of the initial DON concentration and treatment time on DON degradation were carried out. Possible DON degradation compounds were evaluated and its toxicity was *in silico* estimated.

2 Materials and methods

2.1 Chemicals

DON was bought from Romer Labs (Tulln, Austria). Methanol HPLC grade, acetonitrile HPLC gradient grade and sodium chloride were from Fisher Scientific UK Limited (Loughborough, UK) and NH_4OH was bought from Scharlab (Barcelona, Spain).

2.2 Samples and kernel contamination

Wheat kernels ($11.24 \pm 0.01\%$ moisture content), with a DON contamination below $11.3 \mu\text{g/kg}$ (LOD of the method), were kindly donated by Aragonesa de Harinas S.A. (Regany group). DON-contaminated wheat kernels were prepared by adding an aqueous solution of DON to DON-free wheat kernels. To do that, 12.5 g of wheat kernels were put in a 50 ml Falcon tube, and 0.5 ml of DON standard aqueous solution were added to them by pipetting $50 \mu\text{l}$ of solution a total of 10 times, closing the tube and agitating it between additions to ensure an homogenous distribution of the toxin amongst the kernels. Final moisture

content of the kernels increased by approximately 4% (0.5 ml added to 12.5 g).
This procedure was repeated for each fortified sample.

2.3 System for ammonia treatment of kernels

To study the effect of NH_3 treatments on DON-contaminated wheat kernels, the structure sketched in **Fig. S1** (in supplementary material) was designed. Briefly, in a 430 ml canned food glass jar, a glass Petri dish containing NH_4OH solution (2 ml) was placed at the bottom. The DON contaminated wheat kernels (12.5 g) were placed on a wire mesh 5 cm above the Petri dish allowing the contact with the NH_3 vapours on the headspace of the jar. The glass jar was hermetically sealed with a jar lid. The jar was heated in a hot air oven (JP Selecta 210, JP Selecta S.A., Abrera, Spain) for a specific temperature and time. After the heat treatment, wheat was left to aerate in a laboratory fume hood for 15 min to remove residual ammonia. Samples were weighted after the treatment in order to control moisture content variations. DON was analysed according to sections 2.6 and 2.7.

2.4 Experimental design for DON degradation with ammonia

According to the scarce literature and our preliminary tests, temperature and NH_4OH solution concentration were chosen as the factors for the optimization of DON degradation. The selected temperature range (from 65 to 115°C) was based on the experiments of Young (1986) and Weng et al. (1994). The DON concentration of kernels was 500 $\mu\text{g}/\text{kg}$. A 3-level-2-factor central composite design (CCD) with face centered axial points ($\alpha=\pm 1$) and three replicates of the

center point was designed. The conditions for the 11 runs of the CCD and the results for DON degradation (expressed in %) are shown in **Table 1**. Three different temperatures (65, 90 and 115°C) and three different NH₄OH concentrations (1.6, 3.2 and 4.8%) were assayed. NH₄OH concentrations are expressed as % of NH₄OH respect to the wheat sample weight, and were prepared by pipetting 2 ml of 10, 20 and 30% NH₄OH stock solution into the Petri dish. The treatment time was established in two hours based on preliminary tests. All DON degradation values are relative values, and were calculated respect to non-treated samples (reference samples). RSD of reference samples was <2.8%. In addition, negative controls for each temperature (analysis of the DON-contaminated wheat, replacing NH₄OH solution by water) were conducted. Reference samples and negative controls were done in duplicate.

Response surface methodology was used to model and optimize DON degradation (%) according to Eq. (1), where \hat{Y} is the value estimated with the model, and $b_{i,j}$ the regression coefficients (0 is the intercept, 1 is temperature (T) and 2 is NH₄OH concentration) that include the lineal and quadratic effects and the two-way interaction.

$$\hat{Y} = b_0 + b_1 \cdot T + b_2 \cdot \text{NH}_4\text{OH} + b_{1,1} \cdot T^2 + b_{2,2} \cdot \text{NH}_4\text{OH}^2 + b_{1,2} \cdot T \cdot \text{NH}_4\text{OH} \quad (1)$$

Taking into account the slow heat transfer into the jar, the evolution of the sample temperature was recorded during the treatment for each temperature assayed. A thermobutton (Datalogger 22E, Plug & Track, Willems, France) was placed amidst the wheat kernels, registering the temperature every minute. The evolution of the temperature vs. time for each treatment can be seen in **Fig. S2** (in supplementary material). Total transmitted heat to the wheat were 1.17 kJ,

2.14 kJ and 3.24 kJ for 65, 90 and 115°C treatments, respectively. Changes in specific heat of wheat due to different temperature were taken into account (Cao, T., Li, G. , Zhang, Z., Chen, L., Li, Y., Zhang, 2010; Jayas & Cenkowski, 2006).

2.5 Kinetic and effect of toxin concentration studies on DON degradation under optimal conditions

With the optimal DON degradation conditions selected from the data obtained in the experimental design (90°C and 4.8% NH₄OH), studies on the kinetics of DON degradation and on the effect of DON concentration on its degradation were carried out. All obtained DON degradation values are relative values calculated respect to reference samples.

In the kinetic study, eight glass jars with 12.5 g of contaminated wheat kernels (500 µg/kg DON) were prepared, containing each 4.8% NH₄OH. The jars were held at 90°C in the hot air oven and were removed from it one by one in different periods of time (60, 90, 120 and 240 min). Two replicates for each treatment time were carried out.

As for the study of the effect of DON concentration on its degradation, six glass jars were ammonia treated for 2 h at 90°C, each of them containing also 12.5 g of wheat kernels and 4.8% NH₄OH. The different DON concentrations tested were 200, 500 and 2000 µg/kg. Tests were performed in duplicate.

2.6 DON extraction from wheat kernels

Samples were prepared according to the study of Zhang et al. (2019) with some modifications. The 12.5 g of wheat were ground in a IKA A11 (IKA®-Werke GmbH & Co. KG, Staufen, Germany) mill during 30 s. 5 g of ground wheat were transferred into a 50 ml Falcon tube, and 1 g of NaCl and 40 ml of milli-Q water were added. The mixture was vortexed for 15 s and ultrasound-treated with the Branson M2800H-E (Branson Ultrasonic SA, Carouge, Switzerland) at maximum power during 15 min. After that, the Falcon tubes were centrifuged in a Hettich 320R centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) at 8965g for 10 min at 20°C. DonPrep immunoaffinity columns (Biopharm AG, Darmstadt, Germany) were prepared by adding 10 ml of milli-Q water. 8 ml of the supernatant were collected and passed through the immunoaffinity column. After that 1.5 ml of methanol were added to collect the toxin. Backflushing was done three times, and then other 0.5 ml of methanol were passed through the column. The 2 ml of collected methanol were evaporated at 40°C (Stuart SBH200D/3 block heater, ©Cole-Parmer, Staffordshire, UK) under a gentle stream of N₂. The residue was re-suspended in 1 ml of MeOH:H₂O 10/90 (v:v), vortexed, filtered through 0.22 µm PTFE filters and analysed by HPLC-DAD according to section 2.7.

2.7 HPLC-DAD DON analysis

The determination of DON was performed using an Agilent Technologies 1260 Infinity HPLC system (California, USA) coupled with an Agilent 1260 Infinity II Diode Array Detector (DAD). A Phenomenex®Gemini C18 column (California, USA) was used (150 x 4.6 mm, 5 µm particle size, 110 Å pore size).

Absorbance reading was performed at 220 nm. Three mobile phases were prepared: phase A (100% methanol), phase B (methanol:water 10:90, v:v) and phase C (acetonitrile:water 20:80, v:v). The gradient applied was as follows: 0 min 100% B; 10 min 60% B and 40% C; 13 min 60% B and 40% C; 15 min 100% A; 25 min 100% A; 29 min 100% B until 40 min (including the cleaning and re-equilibrating of the column). Flow rate was set at 1 ml/min. The column temperature was 40°C, and the injection volume 50 µl. DON retention time was 10.2 min. LOD and LOQ, considered as three and ten times the signal of the blank, were 11.3 and 37.6 µg/kg. Quantification was carried out by using DON calibration curves prepared in methanol:water 10:90 (v:v). Recovery was assayed in duplicate at three concentrations (100, 400 and 700 µg/kg). Average recovery values were 92.7 ± 9.7 %. Repeatability and reproducibility of the method were <3.0 % and <16.2%, respectively.

2.8 Study of the possible DON-derived formed products.

To identify the DON-derived formed product/s the following sample was prepared: an amount of DON standard solution containing 4 µg of pure DON was pipetted into an amber glass vial and evaporated at 40°C under a gentle stream of N₂. The dried vial was then treated with NH₃ vapours in a hot air oven like the DON-contaminated wheat kernels in sections 2.3 and 2.4, but putting only the vial instead of the kernels on the wire mesh. The oven temperature was set at 90°C, and 4.8% NH₄OH was added. The vial with DON was treated for a total time of 20 h to ensure total degradation of DON. After that, 1 ml of MeOH:H₂O 10/90 (v:v) was added to the vial to re-suspend the formed products, vortexed, and filtered through 0.22 µm PTFE filters.

After evaluation of the complete DON degradation by injection in the HPLC-DAD system described in section 2.7, the degraded sample, and a non-degraded reference sample, were injected in a liquid chromatographic system coupled to a mass spectrometer (LC-MS). The LC-MS system was a Waters Acquity UPLC equipped with a binary pump, an autosampler and a heated column compartment (40°C). The column was an ACQUITY UPLC® BEH C18 1.7µm (2.1x150 mm) from Waters. The mobile phases were A (water:methanol (50:50)-0.1% formic acid) and B (methanol). The system was operated with a flow of 0.35 ml/min and the gradient applied was as follows: 0 min 100% A; t = 1.5 min 100% A; t = 3 min 100% B; t = 3.5 min 100% B; t = 3.51 min 100% A. Injection volume was 2.5 µL. The detector was an Acquity TQD tandem quadrupole mass spectrometer. The electrospray ionisation (ESI) source was operated in positive mode. ESI parameters were: desolvation temperature, 300°C; desolvation gas (N₂) flow rate, 800 L/h; cone gas (N₂) flow rate, 150 L/h, capillary voltage, 3.5 kV; and source temperature, 150°C. The data were acquired from 250 to 350 m/z (MS1-scan mode and collision energy 5 eV). The tentative identification of the degraded products of DON was carried out by studying the possible chemical reactions between DON and NH₃ and by searching for the compounds in the chromatograms on the basis of their molecular ions [M+H]⁺.

2.9 *In silico* toxicity and biological activity evaluation.

For the evaluation of the differences in toxicity and biological activities of the degraded compounds respect to the parental mycotoxin, two web tools were used. Chemical structures and SMILES notations were generated by using ACD

labs Chems sketch software (version 2018.2.1). Biological activities and Lipinski's rule were calculated by using the Molinspiration software version 2018.03 (www.molinspiration.com). For the biological activities, higher score values indicate higher activity. The biological activities evaluated were: G protein-coupled receptor (GPCR) ligand; ion channel modulator, kinase inhibitor, nuclear receptor ligand, protease inhibitor and enzyme inhibitor. For evaluating the permeability across the cell membrane of the compounds the Lipinski's rule of five were used. This rule establishes that for a good permeability across the cell membrane the compound must meet: a) molecular weight under 500 Daltons, b) octanol/water partition coefficient lower than 5 ($\text{Log } P < 5$), c) less than 5 hydrogen bond donors (nitrogen and/or oxygen), and d) less than 10 hydrogen bond acceptors (nitrogen and/or oxygen). The Lipinski's rule establishes that for a compound to be orally active, it must no more than one violation in this rule (Lipinski, Lombardo, Dominy, & Feeney, 2001). For the evaluation of the toxicity (mutagenic; tumorigenic, irritant, and reproductive system effects) the tool Osiris property explorer software (www.organicchemistry.org/prog/peo/) were used. This program estimates the toxicity as red (high risk), yellow, and green (low risk or drug-conform behavior).

2.10 Statistics

One way-ANOVA and least significance difference (LSD) Fisher tests were used to evaluate the effects of the different treatments and sample types. Significance level was established at 0.05 and confidence limits at 0.95. Statistical analyses were carried out by using STATISTICA program for Windows (version 7.1) (StatSoft, Inc., 2005, www.statsoft.com).

297

298 3 Results and discussion

299 3.1 Effect of temperature and NH_4OH concentration on DON degradation

300 The analysis of the results of the experimental design revealed significant
301 effects (ANOVA, $p < 0.05$) for the linear and quadratic components of both
302 temperature and NH_4OH concentration, but not for the two-way interaction
303 between them. Hence, the model was recalculated leaving out of the equation
304 the two-way interaction for greater accuracy. Results of the ANOVA and
305 regression coefficients are presented in **Tables S1** and **S2** (in supplementary
306 material). Pareto chart is presented in **Fig. 1**, showing the significant effects
307 affecting DON degradation. As can be seen, the strongest effect was caused by
308 the linear component of temperature, followed by the linear component of
309 NH_4OH concentration and the quadratic component of temperature. The
310 quadratic component of NH_4OH concentration was also significant, but had a
311 considerable lesser relevance in comparison to the other components. Linear
312 components of both temperature and NH_4OH concentration had a positive
313 effect on DON degradation, while quadratic components of the two variables
314 had the opposite effect. In the corresponding response surface graph (**Fig. 2**) it
315 can clearly be seen the lineal positive and quadratic negative effects of
316 temperature and NH_4OH concentration. As quadratic components are of lesser
317 importance than lineal ones, DON degradation generally increased with
318 temperature and NH_4OH concentration.

319 According to the model, highest predicted degradations correspond to
320 treatments of 115°C with 4.8% NH_4OH (72.49% degradation) and 90°C with
321 4.8% NH_4OH (71.62% degradation). Taking into account the little difference in

degradation between treatments, and that heating the media from room temperature to 115°C is by far more expensive than doing it only up to 90°C, 90°C with 4.8% NH₄OH could be considered the optimal conditions for DON degradation.

Considering that this treatment applies moderate temperatures, the contribution of thermal treatment to DON degradation was evaluated employing two negative controls (DON contaminated wheat samples heated in the oven but with 2 ml of water instead of 2 ml of NH₄OH solution) for each temperature (65, 90 and 115°C). The DON degradation percentages at each temperature regarding reference samples (negative controls, relative values) can be seen in **Fig. 3**. While the 115°C treatment caused a significant DON degradation (22.05%), the differences between reference samples and 65 and 90°C treatments were not significant. Therefore, the degradation observed in ammonia treated samples is mainly caused by the ammonia effect.

3.2 Kinetic and effect of toxin concentration studies on DON degradation under optimal conditions

DON degradation kinetics obtained with the previously optimized conditions are shown in **Fig. 4**. As can be seen, treating a sample under optimal conditions (4.8% NH₄OH, 90°C) for a total time of 60 min will lead to a 45.73% DON reduction. For 120 min, 77.39% of DON degradation is achieved. Extending the process for another 2 hours will increase total DON reduction up to 92.73%.

Regarding the effect of the initial toxin concentration (ranging from 200 to 2000 µg/kg) on its degradation under optimal conditions (4.8% NH₄OH, 90°C) and

two hours of treatment (**Fig. 5**), no significant differences were observed between DON initial levels on the treatment effectiveness.

Therefore, under optimal conditions and two hours of treatment, more than 75% of DON can be degraded for wheat samples affected with a moderate contamination, probably avoiding the discard of batches of the product. In addition, this treatment followed by proper aeration would minimize the ammonium residues in the sample compared to direct treatments with an ammonium hydroxide solution or with an ammonium salt.

To our knowledge, this is the first study aimed to degrade DON by gaseous ammoniation in wheat kernels. Young et al. (1986) decontaminated DON in wheat but soaking the kernels in liquid ammonium hydroxide (5% concentration, 600 ml/kg wheat) for 24 h at 22°C, achieving only a 35% DON reduction. Other studies using gaseous ammoniation for detoxification of other mycotoxins have been published, yielding similar results to our work. Weng et al.(1994) treated aflatoxin contaminated (354µg/kg) corn (12% moisture) with 2% NH₃ at 17 psi and 121°C, obtaining a 98.6% aflatoxin reduction after a 60 min treatment. Gardner Jr. et al.(1971) observed that treating aflatoxin contaminated peanut meal (121 µg aflatoxin/kg, 9% moisture) with ammonia at 15 psi and 93°C for 30 min, 80.2% total aflatoxin degradation was achieved.

3.3 Study of DON-derived formed products and *in silico* biological and toxicological evaluation.

The identification of the DON degradation products generated by the ammonia treatments in real wheat samples presents a strong analytical difficulty related

to the retention capacity of the immunoaffinity columns, and/or the obtaining of clean samples adequate for the analysis by mass spectrometry. For these reasons, in order to obtain a clear idea of the type of compounds resulting from the reaction of DON and ammonia, several experiments were carried out. These experiments were performed in a similar way of the wheat treatments, by the exposition of a dry vial containing DON to NH_3 vapors at 90°C , obtaining almost a total DON degradation. The HPLC-MS chromatograms, obtained in full scan acquisition mode, are presented in **Fig. S3** (in supplementary information). For the reference sample DON was detected for m/z 297 and at 0.84 min. For degraded sample clear peaks at m/z 296 (peak time 1.06 min), 294 (peak time 0.88 min), and 312 (peak time 1.12 min) were observed, that would correspond to the degraded products molecular ions, being the less intense peak the first one (m/z 296).

Considering the chemical structure of DON, several points of interaction are possible between DON molecule and NH_3 . Thus, the carbonyl group, the epoxide ring and the double bond in α,β with carbonyl group are suitable for a nucleophilic attack of the ammonia. In the case of carbonyl group, the NH_3 addition would be followed of a water elimination to form an imine. On the other hand, the nucleophilic attack to the carbon of the epoxide ring (producing the ring-opening), and the attack to the double bond (via Michael addition reaction), would form an amine in both cases. In addition, the tertiary alcohol formed in the case of epoxide ring-opening could suffer dehydration to form an alkene (Carey & Sundberg, 2008).

Therefore, on the basis of these possible chemical reactions, the peak characterized by the ion m/z $[\text{M}+\text{H}]^+=296$, that correspond to the molecular

mass 295, can be produced by the formation of an imine (compound A in **Fig. S4-Scheme a**, in supplementary material), or by epoxide ring-opening, forming an amine, and dehydration of the tertiary alcohol generated (compound B in **Fig. S4-Scheme b**). On the other hand, the major chromatographic peaks, m/z 312 and 294, require the reaction of multiple ammonia molecules with the DON molecule, being that coherent with the high excess of ammonia respect to the mycotoxin. Thus, the compounds of the molecular ion $[M+H]^+$ 294 would be generated by a first formation of an imine by the reaction of the carbonyl group. After that, the keto-enol tautomerism could produce another carbonyl group and the formation of a second imine group. Both imine groups would be probably enough stable in water solution due to the imine-enamine tautomerism and the conjugated double bonds in the ring. A third addition of ammonia to the ring of epoxide group would produce the ring-opening and the dehydration of the tertiary alcohol generating the compound with molecular weight 293 (compound C in **Fig. S4-Scheme c**). However, the formation of the compound with molecular weight 312 would be explainable by a first Michael addition to the double bond and two subsequent additions to carbonyl groups (compound D in **Fig. S4-Scheme d**).

In **Fig. 6** is shown the chemical structures of DON and the proposed degraded DON compounds. These structures allow to observe that the modifications on the molecule of DON due to the ammonia treatment would be mainly on the epoxide ring and on the carbonyl group and hydroxyl group of the six-carbon ring. In this sense, taking into account that the toxicity of DON is mainly due to the epoxide group (Ehrlich & Daigle, 1987), and that the alteration of the α,β -unsaturated ketone moiety can produce a toxicity decrease (Fruhmann et al.,

2014), it can be assumed that the degraded products generated by the ammonia treatment would be less toxic than the parental molecule. In order to evaluate the possible biological activity and toxicity of the generated compounds, in comparison to DON molecule, two *in silico* tests were carried out by using the tools “Molinspiration” and “Osiris”. In **Table 2** are shown the results for the estimation of biological activities and the toxicity for the compounds A-D and DON. As can be seen, in general, the biological activities and toxicity of the possible degraded compounds are lower than those of the parental mycotoxin. Thus, except for kinase inhibition, the compounds A, B and C showed, for all the biological activities, lower score (less activity) than the original DON. On the other hand, compound D showed lower score as ion channel modulator, nuclear receptor ligand and enzyme inhibitor than DON. Although the compound D showed, in some cases, higher score than DON molecule, for that compound, and also the compound C, a violation of the Lipinski’s rule of 5 (Lipinski et al., 2001) was observed, so its absorption could be limited compared to DON. Regarding to the evaluation of toxicity, the profiles observed were similar to those for biological activities, remarking that all degraded compounds present a lower possible effect on the reproductive system than DON.

4 Conclusions

A novel and simple method for the chemical degradation of DON in wheat kernels, based on reaction with ammonia vapours, is proposed. Results have shown DON degradations higher than 75% in kernels affected by a moderated contamination by exposition to ammonia vapours at 90°C for 2 hours. In addition, the type of compounds generated by the reaction between DON and

ammonia were studied, concluding that the main DON molecule moieties responsible for the toxic effects would be modified generating less toxic compounds. The proposed degradation strategy could be scaled up easily in the industry, not requiring complex and expensive installations. More research is necessary in order to verify, in real wheat matrices, the DON conversion into the proposed degraded products and to evaluate the toxicity of degraded compounds by *in vitro* and *in vivo* assays.

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595 **Supplementary material description.**

596 **Table S1:** Analysis of variance (ANOVA) of the central composite design for
597 DON degradation. Factors included in the model: linear and quadratic
598 components of temperature (T) and NH_4OH concentration ($[\text{NH}_4\text{OH}]$). **Table S2:**
599 Regression coefficient results from the data of central composite design for
600 DON degradation. Factors included in the model: linear and quadratic
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602 Sketch of the structure designed for the treatments of wheat kernels with
603 ammonia. **Fig. S2:** Evolution of the temperature of the sample during ammonia
604 treatments. **Fig. S3:** Chromatograms obtained by HPLC-MS of samples of DON
605 and degraded DON and the spectra of each peak. Spectra were obtained by
606 subtraction of the background spectra to the peak spectrum. **Fig. S4:** Possible
607 chemical reactions between DON and NH_3 according to the peaks and
608 molecular ions detected in the chromatograms of degraded sample. Calculated
609 molecular weight of final products: Compounds A and B: 295.33 g/mol;
610 Compound C: 293.37 g/mol; Compound D: 311.38 g/mol.

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Figure Captions

Fig. 1. Pareto chart of the factors significantly affecting DON degradation. L: linear component. Q: quadratic component.

Fig. 2. Surface plot of the estimated response (DON content reduction (%)) based on the CCD design (effect of temperature and NH_4OH concentration).

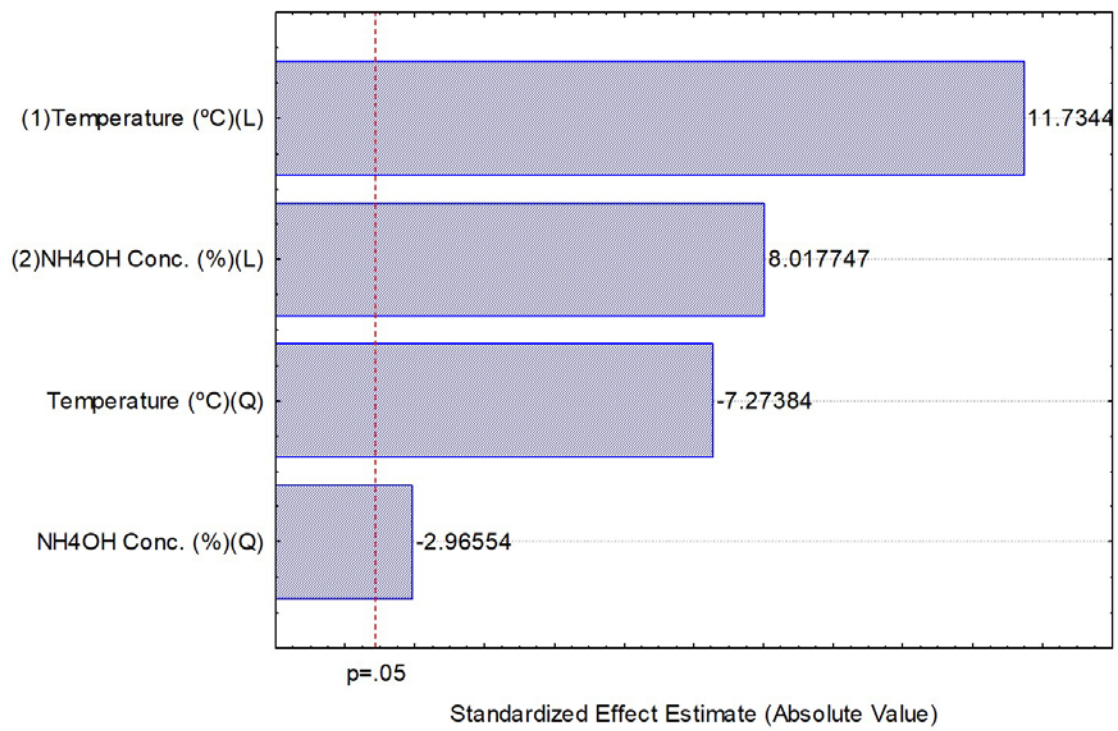
Fig. 3. DON degradation (%) only due to the thermal treatment (negative control). * Indicates statistical differences in ANOVA respect to reference.

Fig. 4. Kinetics of DON degradation (%) by the ammonia treatment under optimal conditions (4.8% NH_4OH , 90 °C).

Fig. 5. Influence of toxin initial concentration on DON degradation under optimal conditions (4.8% NH_4OH , 90°C) and 2 hours of treatment. No significant differences in ANOVA were observed between samples.

Fig. 6. Structures of DON and proposed DON degraded compounds resulted from ammonia treatment. Calculated molecular weight: Compounds A and B: 295.33 g/mol; Compound C: 293.37 g/mol; Compound D: 311.38 g/mol.

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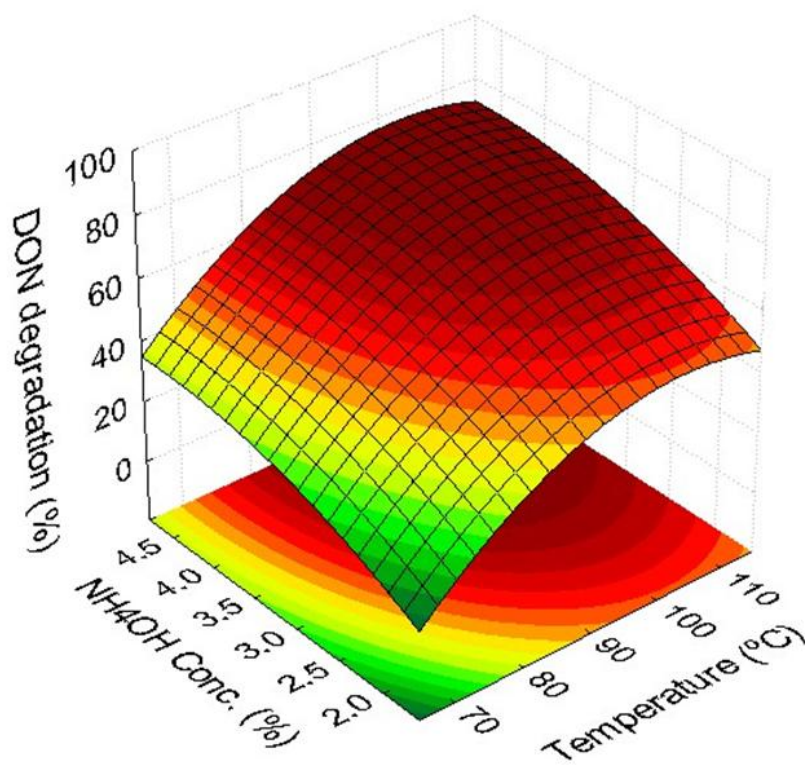
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Figure 1.

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637 **Figure 2.**

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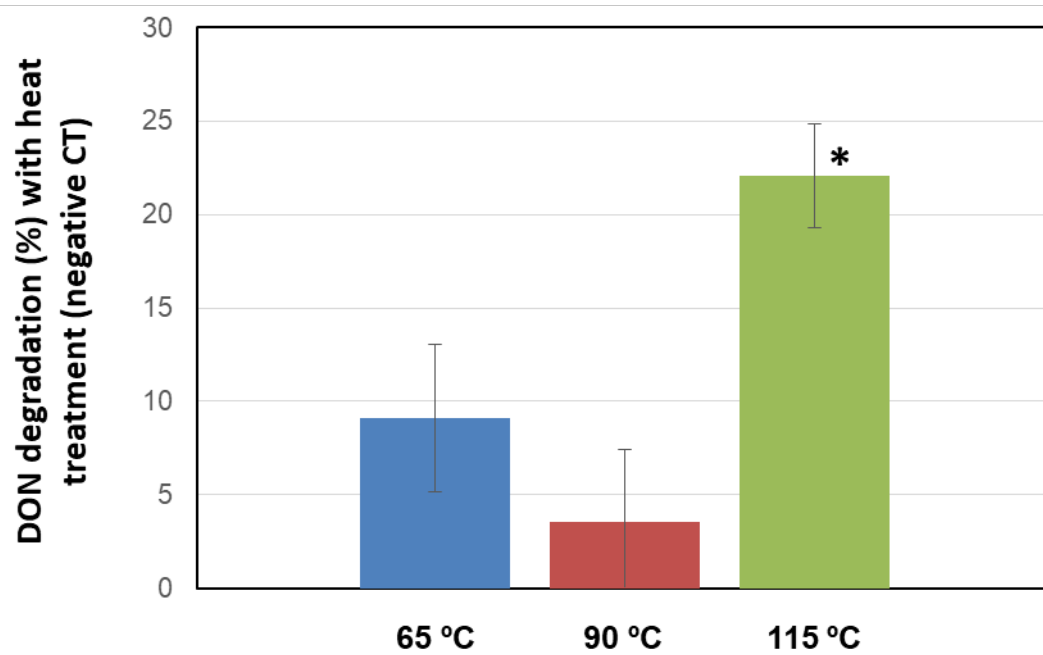
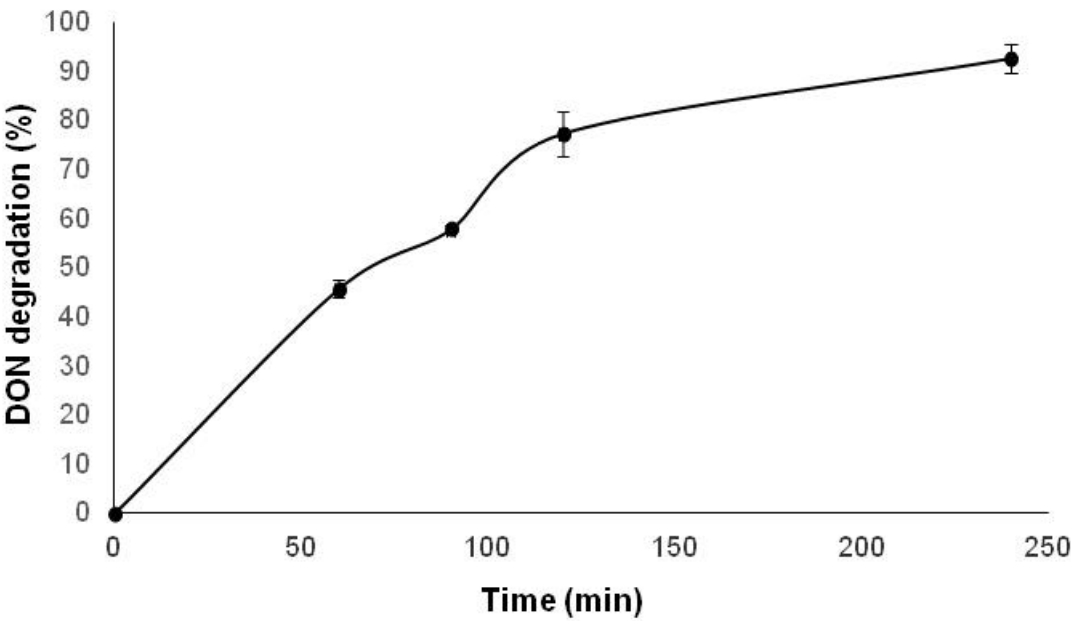


Figure 3.

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Figure 4.

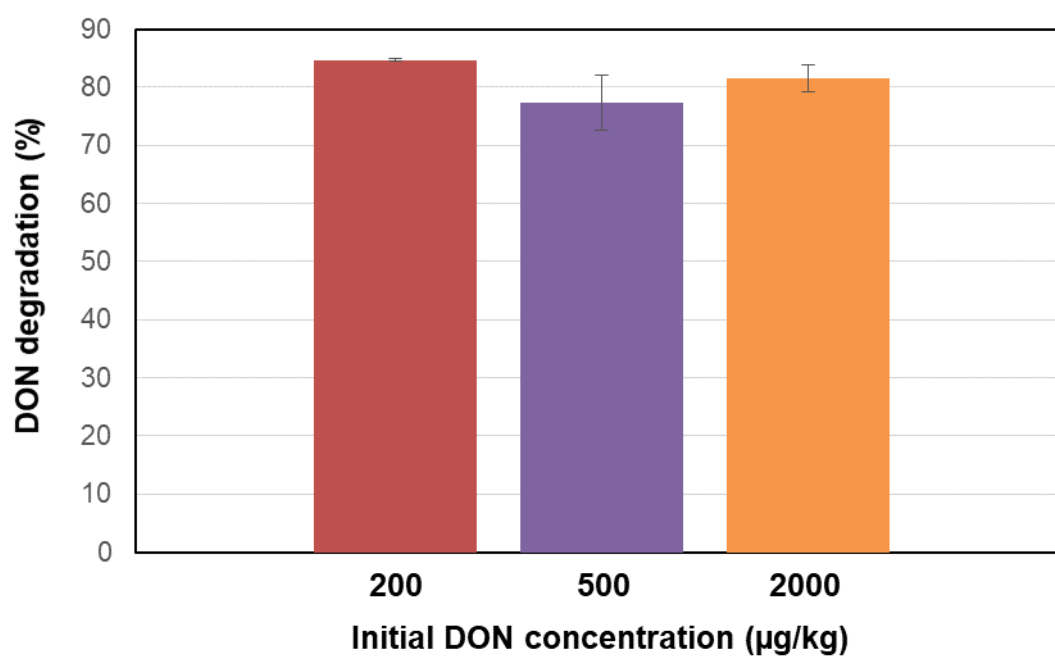
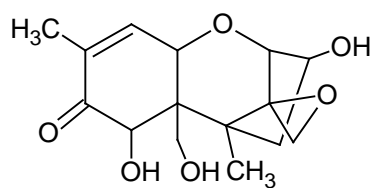
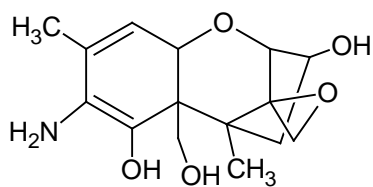


Figure 5.

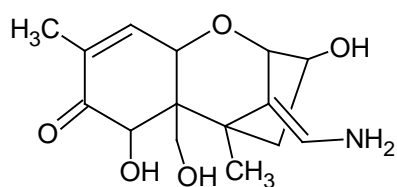
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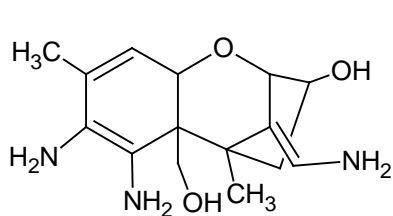
DON



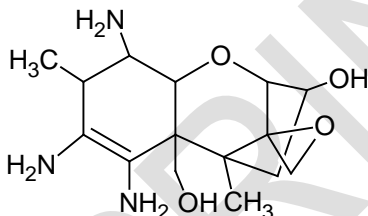
Compound A



Compound B



Compound C



Compound D

Figure 6.

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Table 1. Central composite design 3-level-2-factors with face centered axial points (AP; $\alpha = \pm 1$) and three replicates of the center point (C) for the optimization of the treatment for DON degradation. The factors of the experimental design are: Temperature (Temp) and NH_4OH concentration (%)¹. Samples were fortified with 500 $\mu\text{g}/\text{kg}$ of DON.

Run	Temp. (°C)	NH_4OH Conc. (%) ¹	DON degradation (%)
1	65	1.6	12.78
2	65	4.8	34.56
3	115	1.6	48.95
4	115	4.8	69.73
5 (AP)	65	3.2	25.54
6 (AP)	115	3.2	67.45
7(AP)	90	1.6	39.75
8(AP)	90	4.8	74.57
9 (C)	90	3.2	68.56
10 (C)	90	3.2	66.16
11 (C)	90	3.2	66.62

¹ expressed as % of NH_4OH respect to the wheat sample weight

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Table 2. *In silico* evaluation of biological activities and toxicity.

	DON	Possible Degraded Compounds			
		Compound A	Compound B	Compound C	Compound D
Biological activities ¹					
GPCR ligand	0.09	-0.10	0.07	-0.02	0.17
Ion channel modulator	0.20	0.04	0.10	-0.02	0.01
Kinase inhibitor	-0.60	-0.54	-0.44	-0.30	-0.18
Nuclear receptor ligand	0.66	0.38	0.40	0.22	0.38
Protease inhibitor	0.34	0.18	0.14	0.00	0.65
Enzyme inhibitor	0.50	0.48	0.41	0.38	0.43
Lipinski's rule evaluation ¹					
LogP	-0.97	0.21	-1.35	-0.32	-1.4
Molecular weight (Da)	296.32	295.33	295.33	293.37	311.38
Acceptor H-Bond(nON)	6	6	6	6	7
Donor H-bond (OHNH)	3	5	5	8	8
Violations Lipinski's rule	0	0	0	1	1
Toxicity Test ²					
Mutagenic	red	red	green	yellow	red
Tumorigenic	red	red	green	yellow	red
Irritant	red	red	green	yellow	red
Reproductive system Effect	red	green	green	green	green

¹ Calculated by using Molinspiration software version 2018.03 (www.molinspiration.com). ² Calculated by Osiris property explorer software (www.organicchemistry.org/prog/peo/).

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668 **CRediT author statement**

669 **Bernat Borràs-Vallverdú:** Conceptualization, performed the analysis, writing –
670 original draft; **Antonio J. Ramos:** Conceptualization, writing – review and
671 editing; **Sonia Marín:** writing – review and editing; **Vicente Sanchis:** writing –
672 review and editing; **Juan José Rodríguez-Bencomo:** Conceptualization,
673 writing – original draft.

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POSTPRINT

Title: Deoxynivalenol degradation in wheat kernels by exposition to ammonia
vapours: A tentative strategy for detoxification.

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Sanchis and Juan José Rodríguez-Bencomo*

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Supplementary material description

Table S1. Analysis of variance (ANOVA) of the central composite design for DON degradation. Factors included in the model: linear and quadratic components of temperature (T) and NH_4OH concentration ($[\text{NH}_4\text{OH}]$).

Table S2. Regression coefficient results from the data of central composite design for DON degradation. Factors included in the model: linear and quadratic components of temperature (T) and NH_4OH concentration ($[\text{NH}_4\text{OH}]$).

Figure S1. Sketch of the structure designed for the treatments of wheat kernels with ammonia.

Figure S2. Evolution of the temperature of the sample during ammonia treatments.

Figure S3. Chromatograms obtained by HPLC-MS of samples of DON and degraded DON and the spectra of each peak. Spectra were obtained by subtraction of the background spectra to the peak spectrum

Figure S4. Possible chemical reactions between DON and NH_3 according to the peaks and molecular ions detected in the chromatograms of degraded sample. Calculated molecular weight of final products: Compounds A and B: 295.33 g/mol; Compound C: 293.37 g/mol; Compound D: 311.38 g/mol.

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Table S1. Analysis of variance (ANOVA) of the central composite design for DON degradation. Factors included in the model: linear and quadratic components of temperature (T) and NH₄OH concentration ([NH₄OH]).

	Sum of square	Degrees of freedom	Mean square	F-value	p-value
<i>T</i>	2137.59	1	2137.59	137.697	<0.001
<i>T</i> ²	821.35	1	821.35	52.909	<0.001
[NH ₄ OH]	997.94	1	997.94	64.284	<0.001
[NH ₄ OH] ²	136.52	1	136.52	8.794	0.025
<i>Lack of Fit</i>	89.90	4	22.46	13.852	0.069
<i>Pure Error</i>	3.25	2	1.62		
<i>Total sum square</i>	4452.15	10			

Temperature by NH₄OH linear interaction was excluded due to p value > 0.05. R²=0.9791; R²-Adj=0.9651.

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Table S2. Regression coefficient results from the data of central composite design for DON degradation. Factors included in the model: linear and quadratic components of temperature (T) and NH₄OH concentration ([NH₄OH]).

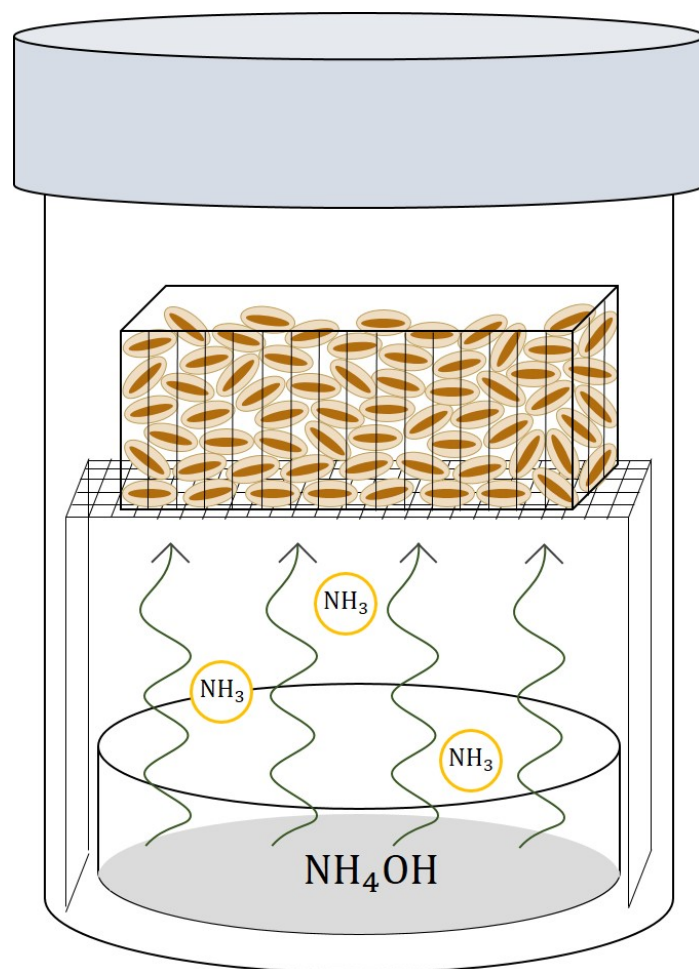
	Regression Coefficients	Standard error	t-value	p-value
<i>Constant</i>	-290.40	30.38	-9.560	<0.001
<i>T</i>	5.94	0.72	8.299	<0.001
<i>T</i> ²	-0.029	0.004	-7.274	<0.001
<i>[NH₄OH]</i>	26.41	6.27	4.213	0.006
<i>[NH₄OH]</i> ²	-2.87	0.97	-2.966	0.025

Temperature by NH₄OH linear interaction was excluded due to p value > 0.05. R²=0.9791; R²-Adj=0.9651.

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Figure S1. Sketch of the structure designed for the treatments of wheat kernels with ammonia.

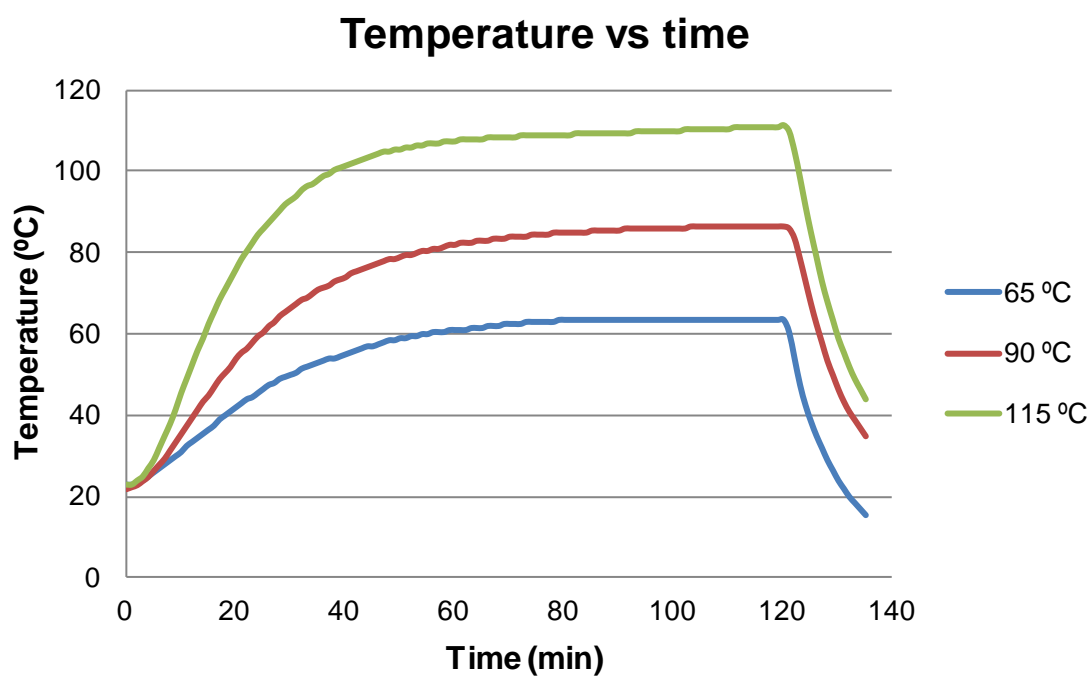


Figure S2. Evolution of the temperature of the sample during ammonia treatments.

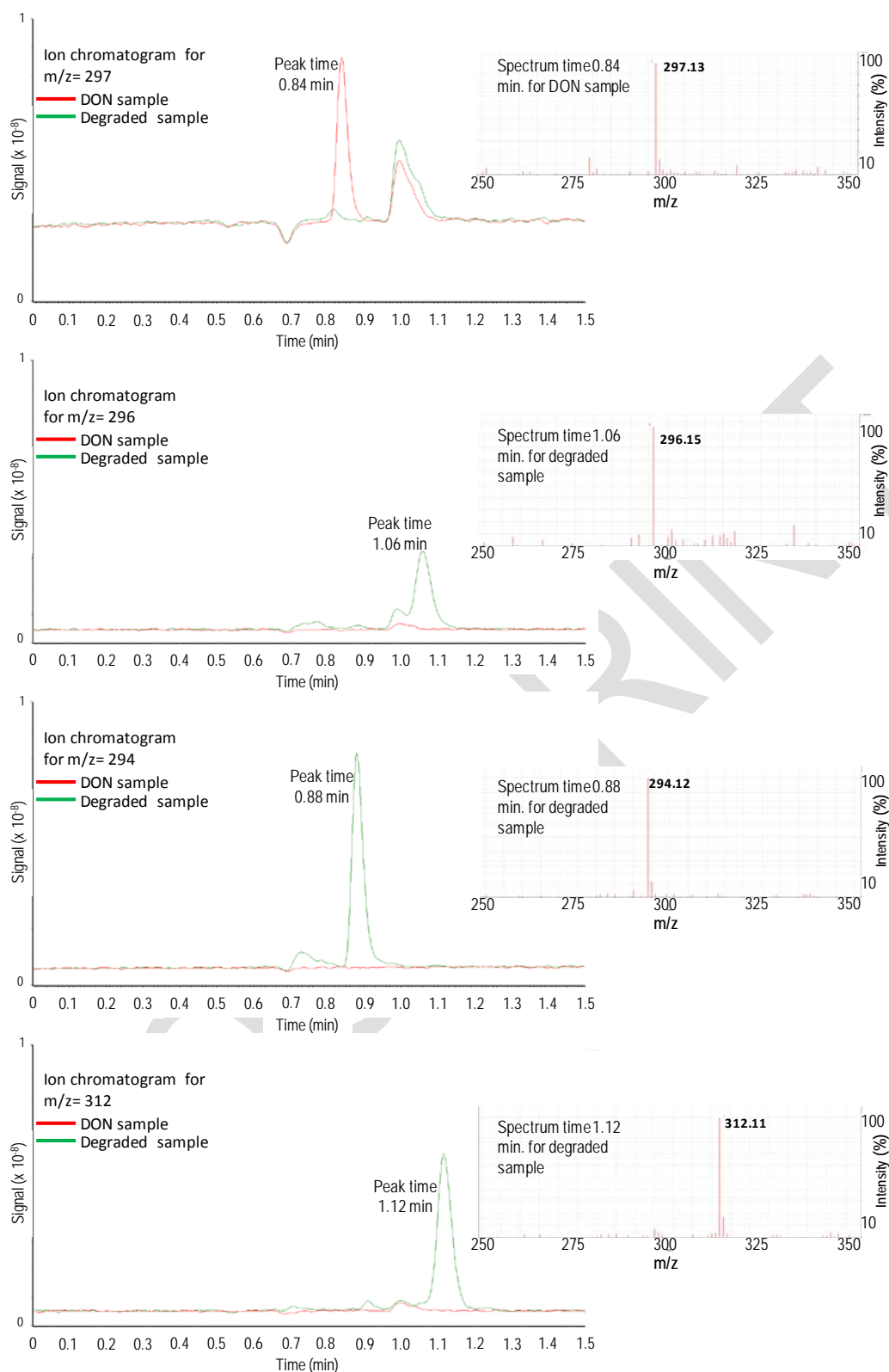


Figure S3. Chromatograms obtained by HPLC-MS of samples of DON and degraded DON and the spectra of each peak. Spectra were obtained by subtraction of the background spectra to the peak spectrum.

Figure S4 - Scheme a

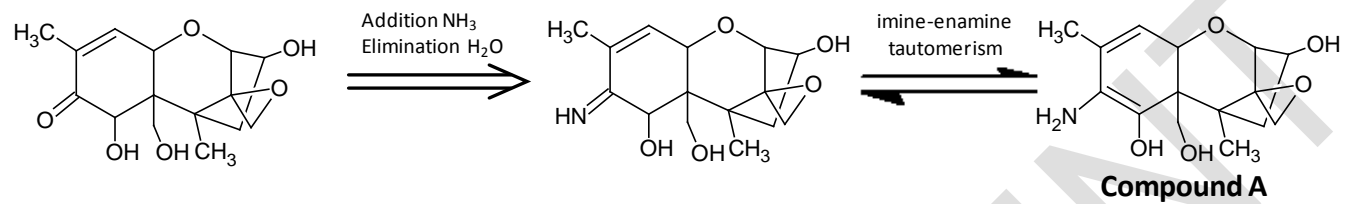


Figure S4 - Scheme b

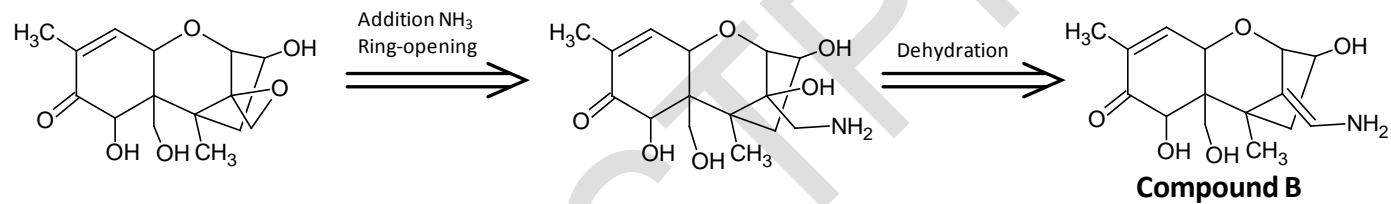
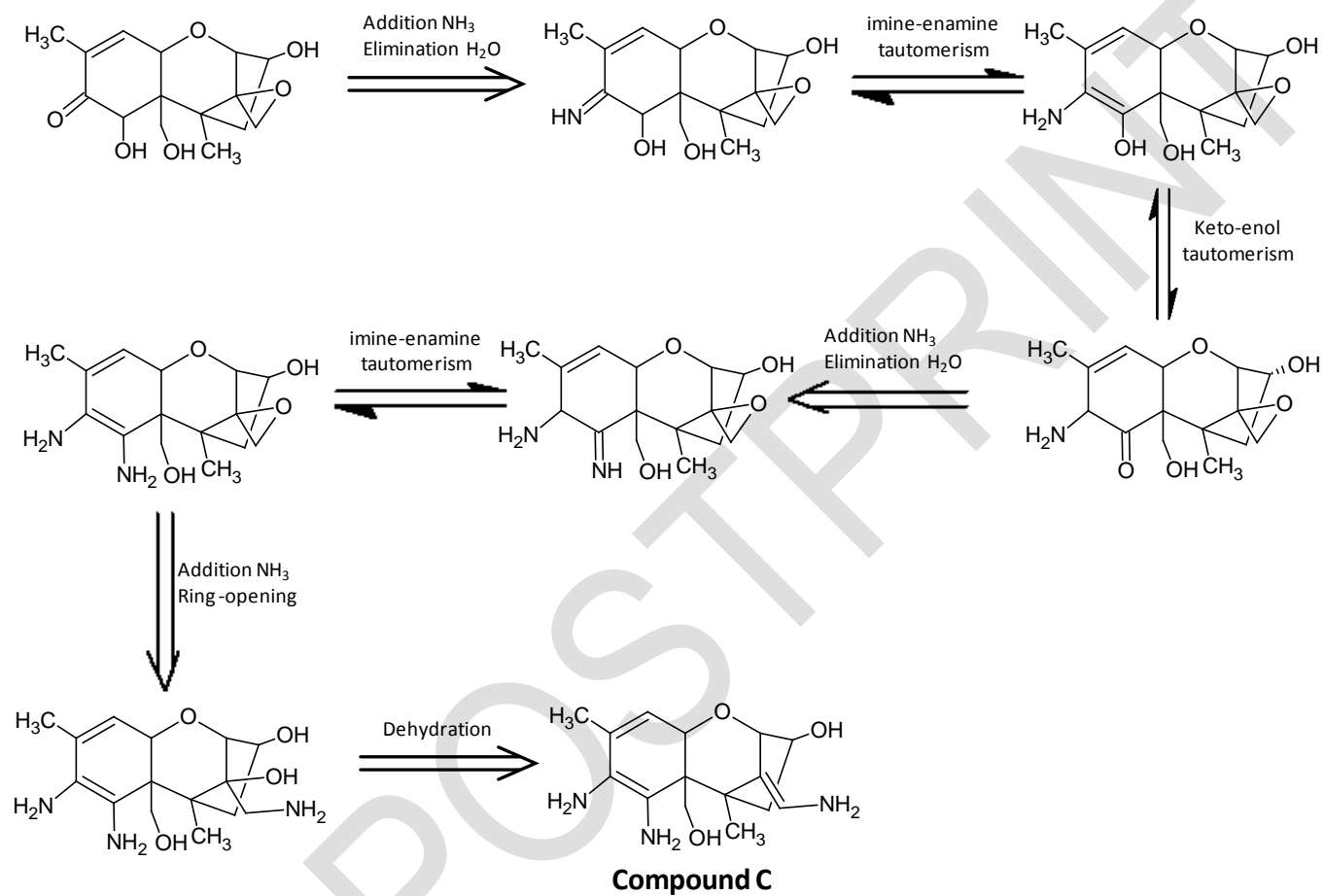


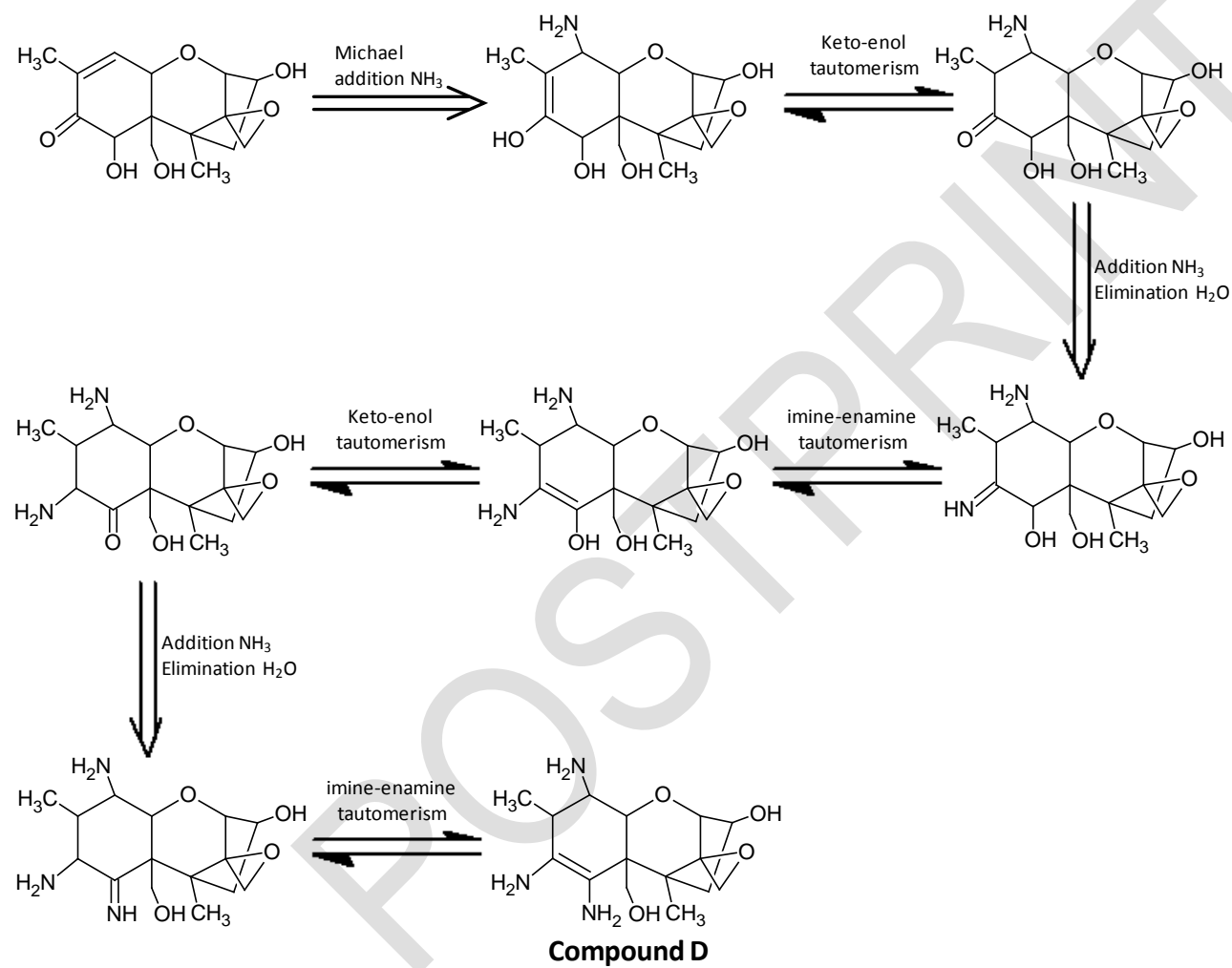
Figure S4 - Scheme c



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Figure S4 - Scheme d



739 **Figure S4.** Possible chemical reactions between DON and NH_3 according to the peaks and molecular ions detected in the
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